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Quaternary structure and function of transport proteins

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Membranes are important sites for the regulation of metabolic functions because they contain transport molecules, which often catalyze the first step in a pathway, and signal-transduction components, which allow the cell to communicate with the environment. Given the catalytic importance of transport proteins and their role in membrane stability, it is possible that oligomerization is used to regulate their function. This review evaluates knowledge of the functions that are associated with the oligomeric organization of secondary transport proteins, which are a major class of solute-translocation systems in all living species.

Membranes are the site of cellular entry and exit of solutes and proteins, and the site of signal transduction. Thus, they represent a prime target for the regulation of cell physiology. Rather than being merely a lipid matrix in which the proteins responsible for these activities float randomly and independently, complex processes involving several components seem to be efficiently organized at and in cell membranes. In addition to roles in transport, and signal and energy transduction, integral membrane proteins are important for retaining membrane architecture, and multimerization of membrane proteins provides a possible mechanism for the specific regulation and stability of membrane structure [1]. From a structural point of view, the majority of membrane proteins could form oligomers as volume exclusion, and protein localization and orientation in the two-dimensional space of the membrane, enhance the likelihood of self-association [2],

In terms of energy-coupling mechanisms, two classes of membrane transporters, the primary and secondary transport systems, are abundant in all known species of eukarya, bacteria and archaea. Primary transport systems convert light or chemical energy into electrochemical energy, such as solute-concentration gradients across membranes. The majority of primary solute-transport systems belong to the ATP-binding cassette (ABC) superfamily. Secondary transport systems use the free energy difference that is stored in the electrochemical gradients of protons, Na^+ ions and other solutes across the membrane to drive translocation reactions. Depending on the direction of transport and whether a coupling ion is used, the systems are termed symporters, antiporters and uniporters. Symporters transport a substrate into the cell (or organelle) against its concentration gradient by cotransport with another solute, most often an H^+ or Na^+ ion, whereas antiporters use the coupling ion (solute) in a countertransport mechanism.

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Secondary transport proteins are composed of a variable number of transmembrane (TM) α-helical segments connected by intra- and extracellular loops (Fig. 1). On the basis of hydropathy profiling, the majority of these transporters, including members of the major facilitator superfamily (MFS), are thought to contain 12 TM α helices. Members of the mitochondrial carrier (MC) family have only six TM α helices and smaller proteins, with as few as four TM α-helical segments, are found in the small multidrug resistance (SMR) family. Because four or six TM α helices are probably insufficient to form a translocation path, these small proteins form oligomeric complexes. As documented for the LacY lactose transporter from *Escherichia coli* [3], the ligand-binding site of secondary transporters is probably an inherent part of the translocation path located within the bundle of TM α helices, rather than a separate domain or protein, as with binding protein-dependent ABC transporters. In the 'alternate access' model of secondary transport, a complete translocation cycle is viewed as the alternating reorientation of the loaded and empty binding site (Fig. 2). Thus, a single binding site is only accessible from one side of the membrane at any given time. The alternate access model accommodates not only solute–ion symport (as depicted in Fig. 2), but also the exchange of solutes at opposing sides of the membrane. In the translocation cycle of alternate access exchange transport, the loaded binding site reorients from outside to inside the cell with one solute, and from inside to outside with another solute. Although the alternate access model conforms to the kinetic mechanism of many symporters, antiporters and uniporters, the transport reaction catalyzed by some systems requires the binding of solutes to two independent sites. In the functional unit (a quaternary structure with a transport function) of these systems, for example the MC family, two substrate-binding sites are present at opposite sides of the membrane and both need to be occupied to complete the translocation cycle.

From studies of two MFS sugar transporters – LacS, the *Streptococcus thermophilus* lactose–proton symporter, and GLUT1, the glucose uniporter in human erythrocytes [4,5] – it is proposed that cooperativity between subunits plays a role in reorientating the empty binding site. Thus, a single subunit composed of 12 TM α helices contains a full substrate-binding site and translocation path, but

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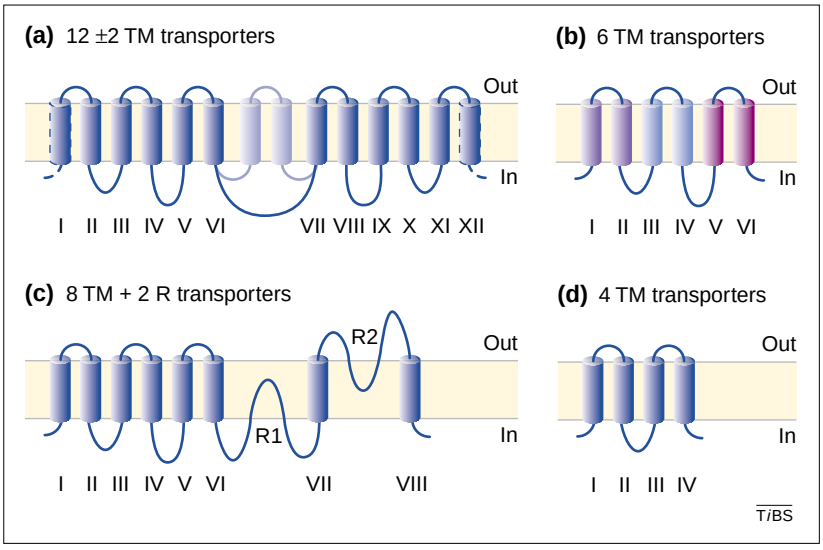

Fig. 1. Membrane topology of secondary transport proteins. Cylinders indicate the positions of the transmembrane (TM) α helices. (a) A subset of 12 ±2 TM proteins have between 10 and 14 TM α helices. A total of 14 TM α helices are indicated. The first and last of these helices (dashed lines) are absent in proteins with 10 TM α helices, which results in the N and C termini facing the outside of the cell. (b) The three-fold repeat sequence of the six TM α helices identified in 6 TM mitochondrial carriers is illustrated by the colors of the first, middle and last pair of TM α helices. (c) The positions of the two reentrant loops (R1 and R2) in 8 TM +2 R transporters (R, reentrant loop) are indicated. (d) 4 TM transporters.

interactions across the oligomer interface control the activity of the system. Other recent information indicates that oligomerization is a general feature of secondary transport proteins. In this review, we consider the ways in which, during evolution, modulation of quaternary structure has been used to regulate the activity, and extend the functions, of the transporters. Experimental determination of the physiologically relevant oligomeric state of a protein is technically demanding, but several complementary techniques can be used to probe the structures of proteins either in detergent solution or embedded in membranes. Because it is accepted that no one method is superior, an overview of the different techniques, with their prerequisites, advantages and shortcomings, is listed in Table 1; some of the more recent techniques are described in more detail below.

Quaternary structure in detergent solution

Although the quaternary structure of membrane proteins is analyzed more easily in the detergent-solubilized rather than membrane-embedded state, the structure of the protein in detergent might not be biologically relevant. Generally, enzyme activity is a good indicator of structural integrity. However, in the case of detergent-solubilized secondary transport proteins, the best that can be measured is ligand-binding activity. Accurate measurement of the amount of detergent (amphiphile) bound to the protein is also needed to determine the quaternary structure because the mass of the amphiphile can be similar to that of the protein [6,7]. Except for sedimentation equilibrium [8], all the techniques listed in Table 1 are nonequilibrium methods. Therefore, it is possible that the oligomeric complexes might dissociate during analysis. Whether oligomeric species are then observed depends on the strength of the protein–protein interactions, which can be influenced by the type of detergent used to solubilize the protein [7,9].

Quaternary structure in the membrane

The oligomeric state of a membrane protein is best determined when it is embedded in the membrane. However, low expression levels and interference by endogenous proteins complicate *in vivo* analyses. *In vitro*, there are the problems of membrane reconstitution, which can be inefficient, and correct insertion of the protein into the membrane, which can be difficult to achieve. The dimensions of a protein in the membrane can be estimated from high-magnification electron-microscope images, the rotational mobility of the molecule, and multicolor colocalization experiments.

By comparing the projection structures of two-dimensional crystals of the TM α-helical proteins opsin, aquaporin 1 and a connexin, Eskandari and co-workers conclude that the cross-sectional area of freeze-fracture particles of α-helical membrane proteins varies linearly with the number of TM α helices [10]. On average, each TM α helix occupies 1.4 nm^2 (Table 2). This value has been used to estimate the quaternary structure of membrane proteins that contain a known number of TM α helices in each subunit. The freeze–fracture technique involves rapid freezing, followed by fracture of the membrane and shadowing with metal-carbon to replicate the specimen. One drawback of this technique relates to the thickness of the metal film, which can be as thick as the difference between the diameter of a monomeric and dimeric 12-TM α-helix subunit [7,10–12]. Problems can also arise if the secondary structure differs from the assumed α-helical organization, such as occurs with 'reentrant loops' (elements that line water-filled cavities but do not span the membrane) [13].

The motional time scales of measurements using saturation-transfer electron-spin resonance (ST-ESR) and phosphorescence anisotropy (10^{-6}–10^{-3} s) are appropriate to observe the rotational mobility of proteins in membranes. Interpreting the data in terms of rotation about the membrane normal requires that the label attached to the protein adopts a unique orientation (i.e. the mobility of the label in the protein must be highly constrained) as, under these conditions, the mobility of the probe reflects the rotational mobility of the protein in the membrane. The probe can be a nitroxide spin label in ST-ESR whereas a suitable fluorophore is used in phosphorescence anisotropy, each attached to a unique cysteine in the protein. The effective radius of the protein can be derived from the rotational diffusion coefficient, provided the viscosity of the

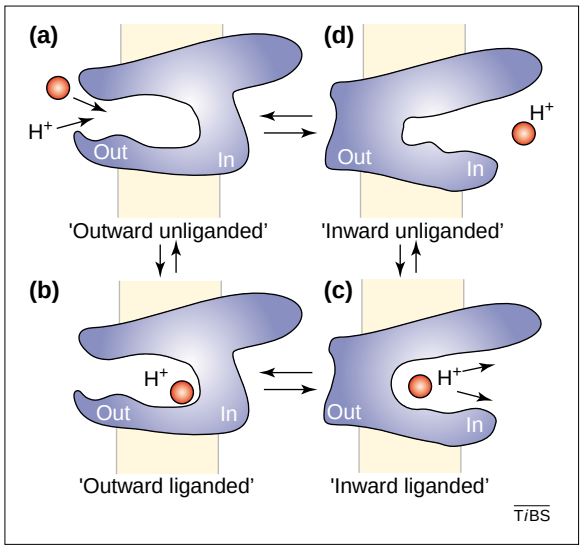

Fig. 2. Alternating access model of secondary transport. Solute–proton symport via alternating access to the ligand-binding site is shown. The cycle depicts proton-motive-force-driven solute uptake. (a) Solute (red circle) and a proton bind to the 'outward unliganded' conformation to form the 'outward liganded' form (b). The liganded binding site then isomerizes from outward to inward to form the 'inward liganded' form (c). The solute and proton dissociate on the inside of the membrane and the 'inward unliganded' form (d) isomerizes from inward to outward, thereby resetting the system for another translocation cycle. If excess substrate is present intracellularly (not shown), the substrate plus a proton can bind to the 'inward unliganded' conformation and the loaded binding site isomerizes from 'inward liganded' to 'outward liganded'. Subsequently, the second substrate and proton will dissociate on the outside of the membrane and the system is primed for the uptake of the next solute molecule. The net reaction of the two half-cycles corresponds to the uptake of one solute and the exit of substrate initially present on the inside. This mode of translocation is referred to as exchange or counterflow transport.

membrane is known [14,15]. The rate of rotational motion is related to the size (effective volume) as well as to the shape, and assumptions must be made to accommodate these parameters. Membrane viscosity can be estimated by calibration with a membrane protein of known shape and structure [15].

Fluorescence-correlation spectroscopy (FCS) allows determination of the diffusion of single molecules in the membrane. Recent advances in reconstituting membrane proteins in giant unilamellar vesicles (diameter 10–100 μm) should make it possible to study the association and dissociation of membrane proteins in native-like bilayers [16]. Although the reconstitution technique has not been used to study the structure–function relationships of membrane-transport proteins in artificial membranes, it could be used in FCS, fluorescence-resonance-energy-transfer and ultrahigh-resolution multicolor colocalization of fluorophore-labeled proteins [17].

Function-related quaternary structure

Whether the monomeric or oligomeric structure represents the minimal functional unit can be deduced from the activity of a system after coexpression of mutant alleles or coreconstitution of mutant proteins. This type of analysis can be performed using either separate genes or proteins, or by fusing genes in tandem. Using this system, the presence of a nonfunctional mutant will not affect the activity of a wild-type protein that is fully functional as a monomer. However, if two or more subunits form the active molecule, the presence of the nonfunctional mutant will decrease the activity. A prerequisite of these studies is the availability of a suitable pair of mutants that have either complementary or a functionally dominant phenotype (either negative or positive) when different species form the hetero-oligomeric complex. In principle, the exact functional unit can be determined from the extent of complementation or functional dominance; that is, the relationship observed between the activity and the ratio of complementary or functionally dominant mutants [4,18,19]. Usually, however, the concentrations of expressed or reconstituted proteins cannot be determined accurately enough to distinguish, for example, between dimers and trimers. The random formation of hetero-oligomers between wild-type and mutant subunits is essential in these experiments and depends on the equilibrium between monomers and oligomers of both subunits. Although there is little information on the equilibrium between monomers and oligomers of transport proteins in the membrane, the tendency to oligomerize seems to be stronger in the membrane than in detergent solution [2,7,15]. If true, this probably reflects a low rate of exchange of subunits between oligomeric complexes in the membrane, and hetero-oligomers should be formed before the insertion of the protein into the membrane. Thus, in coreconstitution experiments, the hetero-oligomers should be formed with detergent-solubilized protein. Hetero-oligomers are formed most readily when the association constants of the interacting species are sufficiently low (K_A <1 μM), to allow mixing at practical concentrations. Varying the concentration and type of detergent can alter the association constants, as determined using analytical ultracentrifugation [6,7,9].

Controls for assessing correct membrane reconstitution or expression are best performed using a cysteine mutant that is inactivated by modification with a thiol-specific reagent. In this case, activity can be measured before and after modification [4]. If functional dominance is not observed using the tandem-fusion approach [18], independent proof that two or more active subunits of two tandem-fusions do not associate to form a functional oligomer should be provided. Similarly, when functional dominance is not observed following either coreconstitution or coexpression of active and inactive polypeptides, the formation of hetero-oligomers should be confirmed before concluding that the functional unit is monomeric.

The minimal functional unit can be also deduced by measuring the activity of proteoliposomes that

Table 1. Techniques to study quaternary structure of membrane proteins in detergent solution[a]

Techniques	Principles and unique features	Prerequisites and drawbacks
Analytical ultracentrifugation	**Sedimentation velocity**	**General**
	In combination with the frictional coefficient, a reasonable estimate of the molecular mass can be obtained	Amount of bound detergent/lipids needs to be determined
	Rigorous quantitative assessment of sample heterogeneity	Sedimentation velocity is dependent on the shape of the protein; does not allow accurate mass determination
	Sedimentation equilibrium	Highly purified protein that is homogeneous and stable for at least a few days
	Independent of protein shape	
	Determination of protein (or protein complex) size; stoichiometry and strength of the interactions between the subunits	
	Determination of protein–protein interactions over a wide range of protein concentrations in a single experiment without perturbing the chemical equilibrium	
Size-exclusion-chromatography	In combination with a sedimentation coefficient, a reasonable estimate of the molecular mass can be obtained	Amount of bound detergent/lipids needs to be determined
		Dependent on protein shape
		Calibration is problematic for membrane proteins
		K_D of the complex needs to be relatively low
Blue native gel electrophoresis	Protein stops migrating when trapped at region of appropriate pore size	Protein solubility and stability in the presence of CBB
	Calibration on soluble proteins is possible when the additional mass of bound CBB is accounted for	Non-equilibrium method
	Many different samples can be analyzed simultaneously	K_D of the complex needs to be relatively low
Co-purification and co-immuno-precipitation	Truncated proteins can be used to define the protein parts that are involved in oligomerization	Hetero-oligomer formation
		Differently tagged protein species are needed
		When association constants in detergent solution are high, these experiments depend on hetero-oligomer formation in the membrane and thus on coexpression in one and the same host

[a]Abbreviations: CBB, Coomassie brilliant blue; K_D, dissociation constant.

are formed at different protein : lipid ratios. As a function of protein : lipid ratio, a linear increase in activity is expected if the functional unit is a monomer, whereas an exponential increase indicates a larger functional unit [19,20]. However, these studies can give a false indication of a monomeric functional unit if strong association in detergent solution leads to incorporation of an oligomeric species at protein : lipid ratios far below the average of one protein per liposome. In addition, measurements of activity are usually difficult to perform and inaccurate if there is an average of <1 monomer per liposome.

Oligomeric state and activity of secondary transport proteins

Our current knowledge of the oligomeric state and activity of secondary transport proteins is summarized in Table 3. Our conclusions concerning the structural and functional oligomeric state of these proteins are based on critical assessment of the literature and take into account the limitations of the different methods and the rigorousness of the published data. Although biophysical approaches that use detergent-solubilized protein are, perhaps, more precise than measurements using membrane-embedded systems, they require highly stable, pure protein, and the quaternary structure observed can be affected by the non-native environment. Functional studies do not reveal the precise oligomeric state but, if biological activity can be related to quaternary structure by, for example, complementation and assays for functional dominance, this type of data are probably most informative. Functional analyses are further strengthened if combined with biophysical methods that determine the quaternary structure in detergent and membranes. Despite uncertainties in the reported analyses, it is clear that the majority of the secondary transport proteins for which the quaternary structure has been studied have an oligomeric organization (Table 3); the relationship between oligomeric structure and function has been established in a few cases.

Is a functional unit of 12 TM α helices the structural paradigm for secondary transport proteins?

Similarities in the sequence of the first and second halves of 12 TM α-helical transporters indicate that these proteins evolved following duplication of a gene encoding a polypeptide with six α helices.

Table 2. Techniques to study quaternary structure of membrane proteins in the membrane[a]

Techniques	Principles and unique features	Prerequisites and drawbacks
Chemical cross-linking	Simple method to demonstrate close proximity of proteins in their native state	Accessibility of target sites Modification of the protein is needed Difficult to exclude aspecific cross-links
Protein–reporter chimera	When coexpressing fusion proteins with different variants of green fluorescent protein, FRET is used to monitor close proximity of proteins *in vivo*	Full-length transcription Correct and functional insertion of the fusion protein into the membrane
Freeze-fracture EM	Determination of the oligomeric state in the membrane Empirically determined 1.4 nm^2/α helix in combination with the predicted number of TM α helices is used to estimate the oligomeric state	Large excess of the protein of interest in the native membrane or incorporation of purified protein into liposomes Secondary structure information is needed Thickness of the metal film needs to be determined
Rotational diffusion	Determination of the rotational mobility of proteins in membranes, from which one can derive the effective radius of the protein	Viscosity of the membranes needs to be determined The label on the protein should solely reflect rotational diffusion of the protein Assumptions about the shape have to be made Correct and functional insertion of the protein into the membrane, or correct and functional membrane reconstitution
Optical microscopy	Determination of single molecule diffusion in the membrane. Possibilities to study the association–dissociation behaviour of membrane proteins in native-like bilayers	Full-length transcription Correct and functional insertion of the protein into the membrane or incorporation of purified protein into liposomes

[a]Abbreviations: EM, electron microscopy; FRET, fluorescence resonance energy transfer; TM, transmembrane.

The two halves, each with six TM α-helical segments, are generally connected by a large cytoplasmic loop (Fig. 1a). In some cases, it is proposed that the cytoplasmic loop gained a hydrophobic character, which caused it to insert into the membrane and resulted in proteins with 14 TM α helices. In other cases, N- and/or C-terminal TM segments seem to have been lost during evolution, resulting in proteins with 10 TM α helices. Because: (1) the majority of secondary transport proteins in the cytoplasmic membrane have 12 ±2 TM α helices; (2) LacY, the prototype 12 TM α-helical protein from *E. coli* functions as monomer [3,14,18]; and (3) dimerization is required for the activity of six TM α-helical mitochondrial transporters [19], it is generally assumed that 12 ±2 TM α helices are required to form a functional translocation path. However, although this is true for the majority of secondary transporters (Table 2), well-studied examples of the systems depicted in Fig. 1c,d do not follow this rule. Moreover, evidence in support of the oligomerization of 12 TM α-helical transporters is increasing, with discrete functions associated with the higher oligomeric structures.

Complete insight into the structural and functional organization of secondary transport proteins needs high-resolution structural information that reveals which residues make up the translocation pathway. Although this detailed information is not available for secondary transport proteins, some medium- and low-resolution projection structures from two-dimensional crystals are available (Fig. 3). Comparison of the projection maps of NhaA, the Na^+–H^+ antiporter from *E. coli* (Fig. 3c), and OxlT, the oxalate–formate antiporter from *Oxalobacter formigenes* (Fig. 3a), at 4 Å and 6 Å resolution, respectively, indicates that both are probably composed of 12 TM α helices, although the packing of the helices is different in the two proteins [21,22]. In both NhaA and OxlT crystals, the molecules are related by a perpendicular two-fold rotational axis, which suggests, but does not prove, that this forms a possible interface for dimeric interaction.

In the projection map of the tetracycline cation–proton antiporter TetA, which is available at 17 Å resolution, the unit cell of the TetA crystals has a threefold symmetry (Fig. 3d), which indicates that the quaternary structure of TetA is trimeric. This is confirmed by single-particle analysis [23]. The oligomeric organization of TetA is consistent with copurification and reporter-fusion experiments (Table 3), which indicates that the oligomeric interactions occur between the first six TM α helices of each subunit [24]. However, data on the functional relevance of trimer formation of TetA are ambiguous and the role of multimerization in protein function remains uncertain [24,25]. Although OxlT and TetA belong to the MFS family, their quaternary structures seem to differ. However, we stress that, except for the projection structures, there is no independent functional or structural information available that supports either a monomeric or dimeric structure of OxlT.

The projection map of NhaA indicates that the translocation pathway lies within the monomer [21]

Table 3. Quaternary structure and functional unit of secondary transport proteins[a]

Family name of secondary transporter[b]	Protein	Species	Number of predicted TM α helices	Structural unit[c]	Functional unit[d]	Techniques[e]	Refs[f]
Sugar porter	GLUT1	Human	12	Dimer and tetramer	Dimer and tetramer[g]	bs,cc,ff,sec	[5,35]
Drug–H^+ antiporter	TetA	*E. coli*	12	Trimer		cp,cs,fc,rf	[23–25]
Organophosphate–P_i antiporter	UhpT	*E. coli*	12	Monomer	Monomer[h]	rt,sec	[20]
Oligosaccharide–H^+ symporter	LacY	*E. coli*	12	Monomer	Monomer[i]	auc,cs,fd,ff,rd,rt	[14,18,36–38]
Galactoside-pentoside-hexuronide–cation symporter	LacS	*S. thermophilus*	12	Dimer	Monomer and dimer[g]	auc,bnp,fd,ff,rd	[4,6,7,15]
	XylP	*Lb. pentosus*	12	Dimer		auc,bnp,ff	[6]
Small multidrug resistance (SMR) porter	EmrE	*E. coli*	4	Dimer of dimers	Tetramer[j]	fd,cs,bs	[31,32]
GDP-mannose–GMP antiporter	LPG2	*L. donovani*	5–10	Oligomer		cc,cp	[39]
	VRG4	Yeast	6–8	Oligomer	Oligomer	cp,fd,sec	[40]
Solute–Na^+ symporter	SGLT1	Rabbit	14	Monomer		ff	[10]
	vSGLT	*V. parahaemolyticus*	14	Monomer		ff	[12]
Neurotransmitter–Na^+ symporter	SERT	Mouse, rat, human	12	Oligomer	Oligomer	cc,cp,fd,fm	[41]
	rGAT-1	Rat	12	Oligomer		fm	[41]
	NET	Rat	12		Oligomer	fd	[33]
	GlyT1/2	Mouse, rat	12	Monomer		auc,bnp	[42]
Dicarboxylate (amino acid)–cation (Na^+ or H^+) symporter (DAACS)	EAAT3	Human	8[k]	Pentamer		cc,ff	[11,43]
	EAAT2	Human	8[k]	Oligomer		cc	[43]
	GLAST	Rat	8[k]	Oligomer		cc	[43]
Citrate–cation symporter	CitS	*K. pneumoniae*	11	Dimer		bnp,cp	[7,44]
Mitochondrial carrier (MC)	AAC	Bovine	6	Dimer	Dimer	auc,bs,cc,sec	[19]
	OGC	Bovine	6	Dimer		cc	[45]
	PiC	Yeast	6	Dimer	Dimer	cp,fd,rt	[19]
Cation–Cl^- cotransporter	NKCC1	Rat	10–12	Dimer		cc	[46]
Anion exchanger	Band 3	Human	8–14	Dimer and tetramer	Dimer and tetramer[l]	auc,bs,cs,sec	[27–30]
NhaA Na^+–H^+ antiporter	NhaA	*E. coli*	12	Dimer	Dimer[g]	cp,cs,fc,sec	[21,26,47]
Monovalent cation–proton antiporter-1	NHE1,3	Rat	12	Oligomer	Monomer[m]	cp,fd,rf	[48]
Phosphate–Na^+ symporter	NaPi-IIA	Rat	8		Monomer[m]	fd	[49]

[a]Abbreviations: *E. coli, Escherichia coli*; EM, electron microscopy; FRET, fluorescence resonance energy transfer; *K. pneumoniae*, *Klebsiella pneumoniae*; *Lb. pentosus, Lactobacillus pentosus*; *L. donovani*, *Leishmania donovani*; *S. thermophilus, Streptococcus thermophilus*; TM, transmembrane; *V. parahaemolyticus*, *Vibrio parahaemolyticus*.
[b]Saier, M.H., Jr (1998) Transport protein classification, http://www.biology.ucsd.edu/~msaier/transport
[c]Structural unit, quaternary structure(s) observed for the detergent-solubilized and/or membrane-embedded state.
[d]Functional unit, quaternary structure shown to be functional in transport.
[e]Techniques used to study the quaternary structure. Abbreviations: auc, analytical ultracentrifugation; bnp, blue native page; bs, binding stoichiometry; cc, chemical crosslinking; cp, co-immunoprecipitation and co-purification; cs, crystal structure; fc, functional complementation; fd, functional dominance; ff, freeze fracture EM; fm, FRET microscopy; rd, rotational diffusion; rf, reporter fusion; rt, reconstitution titration; sec, size exclusion chromatography.
[f]Due to space limitations, we only refer to papers in which the key findings are presented.
[g]Cooperativity between subunits; each subunit can accommodate a translocation path.
[h]Incorporation into liposomes of oligomeric species cannot be excluded.
[i]The majority of evidence points towards a functional monomer, but *in vivo* complementation studies suggest that the protein functions as an oligomer.
[j]Functionality of dimer cannot be rigorously excluded.
[k]In addition to eight TM α helices, there are two reentrant loops.
[l]Dimer is sufficient for transport; additional functions are associated with tetrameric species.
[m]On the basis of lack of negative dominance in *in vivo* complementation experiments.

and the dimer has been proposed to have a role in regulating activity of this Na^+–H^+ antiporter. The activity of NhaA is dependent on pH, as expected for a carrier that is involved in regulating intracellular pH. Coexpressing two mutants with different pH dependencies creates a new Na^+–H^+ antiport activity, with a pH dependence different from that of the individual mutants [26]. Intersubunit cross-linking of a single cysteine mutant also alters the pH profile of NhaA and provides initial structural information of the site(s) at which oligomerization could regulate Na^+–H^+ antiport activity.

The projection map of Band 3, a human anion exchanger, at 20 Å resolution shows a dimeric organization in which the overall shape of the transport domain is unlike that of other membrane proteins. On the basis of the projection map, it has been suggested that the anion pathway consists of a channel located at the dimer interface [27]. However, because the resolution is low, the evidence for this model is weak. Ligand-binding studies indicate that the two subunits interact allosterically [28]. Changes in the oligomeric state of Band 3, from dimer to tetramer to higher oligomer, seem to play a role in

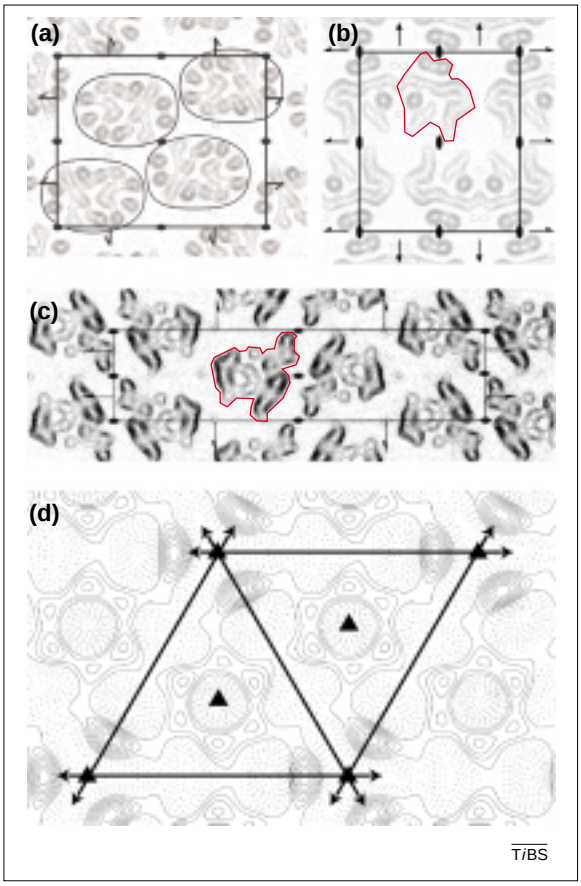

Fig. 3. Projection-density maps of secondary transport proteins. Single unit cells with their symmetry elements are depicted. The projection structures of OxlT, EmrE and NhaA have been normalized to the same scale. (a) OxlT from *Oxalobacter formigenes* at 6 Å resolution; individual proteins are outlined. (b) EmrE from *Escherichia coli* at 7 Å resolution; a putative dimer is outlined in red. (c) NhaA from *E. coli* at 8 Å resolution; a putative subunit within the dimer is outlined in red. (d) TetA from *E. coli* at 17 Å resolution; threefold axes of symmetry are indicated by triangles; a putative TetA trimer is present within each triangle. Figure is reproduced, with permission, from Refs [22,23,31,47].

regulating the multiple functions of this protein, which include anion exchange, stabilization of erythrocyte membrane structure, binding of hemoglobin and glycolytic enzymes, and a role in senescence [1,29,30]. The dimeric state is required and sufficient for anion transport. Tetramers interact with the cytoskeleton via the cytosolic domains of the proteins, thereby contributing to membrane stability. Clustering of Band 3 into higher oligomers occurs in aged and/or damaged cells and triggers the binding of antibodies, which results in removal of these cells.

The EmrE transporter is much smaller than the proteins described above. The projection map at 7 Å resolution shows that EmrE forms an asymmetric dimer (Fig. 3b). Within the dimer there is no obvious twofold axis of symmetry perpendicular to the membrane, indicating that the two subunits have different structures [31]. Comparing two different crystal forms indicates that, in both cases, the crystallizing unit is a tetramer. However, the distance between the helices at the interface of the two dimers is large to allow direct helical contact. Analysis of substrate binding in functional dominance experiments, and copurification of differently tagged species, indicates that EmrE is organized as a dimer of dimers [32]. Although the reported data are most consistent with a model in which the tetramer facilitates transport, they do not exclude the possibility that the dimeric species is functional.

As with NhaA, where dimerization is required for pH-regulated transport activity, there are other examples in which multimerization affects the function of proteins, even when the individual subunits seem to contain a full translocation pathway. For LacS from *S. thermophilus*, cooperativity within the dimer is observed in transport that is driven by proton-motive force but not by equilibrium exchange [4]. It has been proposed that the LacS subunits are functionally coupled in a step unique to the proton-motive-force-driven uptake cycle, most likely a conformational change associated with reorientation of the empty binding site. This implies that the two subunits of a LacS dimer are in opposite conformations at any one time, as has been proposed for the GLUT1 glucose uniporter. The cytochalasin B-binding capacity doubled, and allosteric interactions between substrate-binding sites on adjacent subunits were abolished, as GLUT1 was converted from tetramer to dimer [5].

Another possible aspect of oligomerization is that expression of alternative splice products could provide a mechanism that regulates the activity of oligomeric transport proteins, as has been postulated for the rat noradrenaline transporter and glutamate transporters in human brain [33,34].

Conclusion

Although a seemingly simple and trivial problem, it is very difficult, experimentally, to rigorously address the oligomeric state of membrane transport proteins and the functions of these different states. Nonetheless, it appears that modulation of the quaternary structure of secondary transporters can regulate their activity and function. Among the secondary transporters, there is variation in the quaternary structures, the number of TM α helices that partition in the functional unit, and the function of the oligomers. The minimal number of TM segments needed to fulfill the transport function seems to be 12 ±2, but this does not necessarily mean that the functional unit of twelve, six and four TMS proteins is monomeric, dimeric and trimeric, respectively. Additional functions seem to be associated with proteins that have a quaternary structure that is larger than seemingly needed for the translocation function. There are exceptions to the 12 ±2 rule within the SMR and DAACS families because these transporters consist of protein oligomers of four TM α helices (SMR) and eight TM α helices plus two reentrant loops (DAACS).

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